



Armed Forces Radiobiology Research Institute

**Treatment of Experimental Acute
Radiation Disease in Mice with
Probiotics, Quinolones, and
General Gnotobiological Isolation**

Russia State Medical University

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Preface

Acute Radiation Syndrome, as taught by the Armed Forces Radiobiology Research Institute (AFRRI) in its Medical Effects of Ionizing Radiation course, may be divided into three subsyndromes: hematopoietic, gastrointestinal (or GI), and cardiovascular/CNS syndrome. The range for hematopoietic syndrome is considered to be 1–6 Gy, roughly. (Divisions between these subsyndromes are not absolute, and particularly as the exposure increases, the patient may manifest symptoms characteristic of two or even all three of them.) At the lower end of this range, most people survive with supportive medical care plus antimicrobial treatment. The LD_{50/60} (median dose for survival for 50% of the population at 60 days postexposure) for untreated individuals was considered to be approximately 3 to 3.5 Gy midplane. With modern therapy, including administration of cytokines; granulocyte-macrophage colony stimulating factors; strict isolation techniques; combined antibacterial, antiviral, and often antifungal therapy; fluid and electrolyte therapy; and administration of blood components (irradiated prior to administration to prevent graft vs. host reaction), the LD_{50/60} has now been increased to 5–6 Gy. Cause of death is sepsis aggravated by hemorrhage and depletion of white blood cells.

The GI syndrome is considered to occur between 6 and 30 Gy. With exposure to radiation in this range, the intestinal crypt cells are severely depleted. The mucosal lining of the microvilli is sloughed due to lack of replacement cells. Lymphocytes in the Peyer's patches are destroyed. Edema of the submucosal and muscularis mucosae layers develops, and there is pooling of the microvasculature. Under these conditions, intestinal microflora, including potential pathogenic aerobic and anaerobic bacteria, easily translocate to the mesenteric lymphatic and blood vessels and are transported to the liver and elsewhere in the body. The main cause of death at Chernobyl was sepsis, attributable in many cases to bacterial translocation past the impaired intestinal barriers.

Prevention of intestinal microflora translocation and subsequent sepsis has centered around selective microbial decontamination of the gut to prevent overgrowth of pathogenic organisms. The use of nonpathogenic organisms to compete with and suppress the growth of pathogens has also been studied by the authors of this report. The senior author has published extensively in the fields of changes in intestinal microbial populations following irradiation and how these organisms translocate, antibiotic treatment, selective gut decontamination, general gnotobiological isolation (germ-free environments), and treatment of irradiated animals with antibiotics, immunoglobulins, and nonpatho-

genic anaerobes such as *Bifidobacterium* and *Lactobacillus*, and also suppression of gastrointestinal pathogens.

Indeed, the author and colleagues used this therapy on some firefighters at Chernobyl. Five patients were treated with systemic ampicillin and gentamicin and oral nystatin commencing 4-7 days after irradiation. Three patients also received a strain of *Bifidobacterium longum* for 30 days. Fecal sample measurements showed that the flora were dominated by opportunistic pathogens in the two patients not receiving this preparation, but not in the three who did. One of the two control patients died within the first month postexposure, while the others lived between 4 and 23 months. Although the patients received nonuniform doses of radiation and other physical parameters were also not strictly comparable, the data do indicate that intestinal growth of opportunistic pathogens was suppressed. Clearly, supporting studies in animal models need to be done.

The objective of this project was to study the effects of antibiotics and probiotics (*Bifidobacterium* and *Lactobacillus*) in mice irradiated with 7 Gy. The effects were studied in normal mice and mice raised in total gnotobiological isolation (germ-free environment). This work did demonstrate the effectiveness of *Lactobacillus* in suppressing gram-negative enteric organisms and reducing the translocation of strict anaerobes. This work supplements AFRRRI endeavors in this field and definitely demonstrates the necessity for future research in this area. Prevention of sepsis from the patient's own gastrointestinal organisms is one of the next major hurdles in the treatment of acute radiation syndrome, and the authors have made significant steps toward overcoming this problem as demonstrated in the following article.

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AFRRRI

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Abstract

The effect of ciprofloxacin, lomefloxacin, amikacin, and probiotics (*Lactobacillus acidophilus* 5/4, *Bifidobacterium longum* 44) on intestinal microflora, translocation, and mortality was studied in mice treated with 7.0 Gy radiation.

Lactobacilli and bifidobacteria, selected by *in vitro* and *in vivo* methods, increased survival parameters of

the mice. The effect of lactobacilli was higher under gnotobiological isolation. Lactobacilli suppressed the gram-negative enterics and decreased translocation of the strict anaerobes, but not streptococci. Lomefloxacin increased survival, reducing the intestinal counts and translocation of gram-negative enterics, but not streptococci. A lomefloxacin/lactobacilli combination showed effects similar to lomefloxacin alone.

Introduction

Animal studies have demonstrated that ionizing radiation affects the normal state of the intestinal microbial ecology. In these animals, a reduction in the anaerobic microorganisms, particularly bifidobacteria and lactobacilli, is accompanied by an elevation in the numbers of aerobic and facultative organisms (enterobacteria, pseudomonas, enterococci, staphylococci, yeasts). The numbers of potentially pathogenic gram-negative aerobic and facultative species elevate, and these microbes appear in the small intestine, adhering to the intestinal wall [1-5]. The radiation-induced suppression of immunity and damage to the intestinal wall [6] permit these organisms, together with gram-positive microorganisms (e.g., streptococci, staphylococci), to readily translocate to the bloodstream and reach other organs, causing postirradiation sepsis [5,7,8].

Treatment with antibiotic drugs prevents infections in irradiated animals [9] and humans [10]. Broad-spectrum antibiotics are less effective in the treatment than selectively decontaminating agents, for example, quinolones [11]. In contrast, the newest quinolone, GI-960, which has better activity against anaerobes, does not increase survival after irradiation [1], and quinolones do not prevent sepsis with gram-positive microorganisms (e.g., streptococci) in irradiated mice [7]. Broad-spectrum low-absorbable antibiotics (aminoglycosides) are able to prolong the lives of irradiated animals but also intensify postradiation changes in the animals' intestinal microflora [4]. Furthermore, administration of drugs with stronger action against strict anaerobes (metronidazole) increases translocation of aerobic and facultative bacteria and decreases survival of irradiated animals [12].

Lactobacilli and bifidobacteria, commonly recognized as part of the normal indigenous intestinal microflora in human and animals, are greatly involved in the

host's defense against infections [9,13,14]. Also, peroral treatment of sublethally irradiated animals with live cultures of lactobacilli or bifidobacteria (probiotic preparations, or probiotics) increases survival of the animals as well as "normalizes" the intestinal microflora composition [2,4]. Combinations of probiotics with aminoglycosides or penicillins lead to a greater increase in the survival rate than administration of the antibiotics alone [15,16].

In our minds, these findings demonstrate (i) the significance of intestinal anaerobic bacteria for prevention of endogenous infections in an irradiated host and (ii) the possibility of combining selectively decontaminating antimicrobials (quinolones) with probiotics (bifidobacteria or lactobacilli) to prevent intestinal overgrowth and translocation of potential microbial pathogens.

Currently, the beneficial effects of probiotics *in vivo* are considered to be a combination of direct antagonistic activities against potential pathogens (production of antimicrobials, competition for nutrients and adhesion sites) [17] with indirect mechanisms, such as stimulation of host immunity [13,18]. Moreover, an effective probiotic should not only be capable of producing antimicrobial substances, but should also be able to survive in the intestinal microenvironment, colonizing the intestine for a long period of time [19]. In this connection, the procedure that we used to select the prospective probiotic strains included tests both *in vitro* (antagonistic activity) and *in vivo* (colonization ability and antagonistic activity) on a model of totally decontaminated (TD) mice.

Exogenous microorganisms can also contribute to infections in an immunocompromized host, so the measures directed to prevention of exogenous contamination (protective environment or germ-free isolation)

were included in the treatment [20]. Gnotobiotic isolation (GBI) alone has no effect on survival rate of irradiated animals [9,21], while its combination with antibiotics [21] or probiotics [22] improves this parameter.

The aim of this work was to study the effect of concurrent use of the quinolones (ciprofloxacin, lomefloxacin) or aminoglycoside (amikacin) with the *Lac-*

tobacillus and *Bifidobacterium* probiotics on survival, intestinal microflora, and microbial translocation in sublethally irradiated mice maintained under GBI. In other words, the objectives were to select the strains of bifidobacteria and lactobacilli best suited to suppression of the gastrointestinal pathogens causing sepsis, and to determine the sequence of feeding these bacteria in relation to antimicrobial therapy and gnotobiological isolation.

Materials and Methods

Animals

Conventional animals. Male CBA/lac (Central Laboratory Animals Farm, Kryukovo, Russia), weighing 18–20 g, were used. The mice were quarantined for 2 weeks. Feed and acidified water were given *ad libitum*. Acidified water was replaced with nonacidified sterile water at the beginning of the experiments. At this stage, mice were transferred to a room with a 12-h light-dark cycle.

Totally decontaminated mice and gnotobiological technique. Decontamination of the mice was performed by oral gavage of 0.2 ml of an antibiotic mixture (amikacin, 10 mg/mouse; ampicillin, 4 mg/mouse; nystatin, 4000 U/mouse; and fluconazole, 0.114 mg/mouse) daily for 4 days. Following washing with antiseptic solution (chlorhexidine, 1% v/v) and introduction into the sterilized gnotobiological isolators (La Calhene, France), the animals continued receiving the antibiotics for 2 days. This procedure achieves transitory intestinal decontamination in mice for 7–10 days [23]. To control the efficacy of decontamination in mice, the fecal samples were inoculated onto Bactofoc (Gidrobioz, Moscow, Russia), MRS agar (Oxoid, Great Britain), Endo agar (Serva, Germany), Staphylococcus agar (Difco), Enterococcus agar (Serva), and Sabouraud agar (Serva) plates.

All experimental procedures were done according to the "Guidelines for Work With Laboratory Animals" from the USSR Academy of Science.

Bacterial Strains and Media

Bacterial strains used in this work are listed in table 1. The cultivation media were Bactofoc for bifidobacteria, MRS for lactobacilli, and BHI broth (Difco, USA)

and BHI supplemented with 1.5% agar (Difco) for enterobacteria, streptococci, staphylococci, and pseudomonas. Stock cultures of microorganisms were maintained in the lyophilized state at -20 °C.

Microbiological Methods

Identification of bacteria. Lactobacilli were identified primarily on the basis of their morphology (gram-positive rods) and absence of catalase activity [24], and, finally, at the species level, with API 50CH strips (Bio Merieux SA, Marcy-l'Etoile, France). Bifidobacteria were identified on the genus level according to their cell morphology and the Fructoso-6-Phosphate Phosphoketolase (F6PPK) test [25] and at the species level with the help of API 50CH strips. The other microorganisms were identified according to their cultural, morphological, and biochemical characteristics [26] using appropriate API20 (API System) strips. For identification of anaerobic microorganisms, the antibiotic susceptibility profiles were determined [26].

During *in vivo* experiments, comparison of lactobacilli or bifidobacteria murine reisolates with the initial strains was performed tentatively by phenotypic identification using the API 50CH system. In some cases, the antibiotic susceptibility patterns were also determined. These identifications were confirmed by plasmid profiling and restriction endonuclease analysis of genomic DNA.

Plasmid profiling. Miniprep plasmid isolation from lactobacilli and bifidobacteria was performed as described previously [27]. During the isolation of plasmid DNA from bifidobacteria, the final concentration of lysozyme was 15 mg/ml. Agarose gels (0.7%) were electrophoresed horizontally in Tris-borate buffer [28] at 40 mA for 3–4 h in the presence of 0.5 µg of

Table 1. Bacterial cultures

Strain	Designation	Relevant characteristics	Source
<i>Lactobacillus acidophilus</i> 6/14	L6/14		murine large intestine
<i>Lactobacillus acidophilus</i> 8/14	L8/14		murine small intestine
<i>Lactobacillus acidophilus</i> 14/14	L14/14		murine large intestine
<i>Lactobacillus acidophilus</i> 20/14	L20/14		murine large intestine
<i>Lactobacillus acidophilus</i> 13γ	L13		murine feces
<i>Lactobacillus fermentum</i> 26γ	L26		murine feces
<i>Lactobacillus fermentum</i> 25	L25		murine feces
<i>Lactobacillus acidophilus</i> 5/4	L5/4	P ^r Tc ^s Str ^r Em ^s plasmid free	human feces
<i>Lactobacillus plantarum</i> 18/4	L18/4	P ^r Tc ^r Str ^r Em ^s plasmids of 8, 9 and 16 kb	human feces
<i>Lactobacillus acidophilus</i> 25/4	L25/4		human feces
<i>Bifidobacterium adolescentis</i> UX	BUX	P ^r Tc ^r Str ^s Em ^s plasmid free	human feces
<i>Bifidobacterium adolescentis</i> VL	BVL		human feces
<i>Bifidobacterium longum</i> S2	BS2		human feces
<i>Bifidobacterium longum</i> 44	B44	P ^r Tc ^s Str ^s Em ^s plasmid free	human feces
<i>Bifidobacterium longum</i> 211	B211		human feces
<i>Bifidobacterium bifidum</i> 213	B213		human feces
<i>Bifidobacterium bifidum</i> 221	B221		human feces
<i>Bifidobacterium bifidum</i> 235	B235		human feces
<i>Bifidobacterium bifidum</i> 1/6	B1/6		human feces
<i>Bifidobacterium adolescentis</i> 4M	B4M		human feces
<i>Escherichia coli</i> K13	<i>E. coli</i>		INA
<i>Klebsiella ozaenae</i> K4	<i>K. ozaenae</i>		NCTC
<i>Staphylococcus aureus</i> 209P	<i>S. aureus</i>		INA
<i>Streptococcus faecalis</i> 775	<i>S. faecalis</i>		NCTC
<i>Pseudomonas aeruginosa</i> 27853	<i>P. aeruginosa</i>		ATCC

P, penicillin; Tc, tetracycline; Str, streptomycin; Em, erythromycin; ^s sensitive; ^r resistant

INA, All-Russian Scientific Research Institute on New Antibiotics, Moscow

NCTC, National Collection of Type Cultures, London, U.K.

ATCC, American Type Culture Collection, Rockville, MD

ethidium bromide per ml. The pattern of DNA bands observed in a bacterial extract when gels were examined by UV transillumination was photographed and deemed to be its plasmid profile. Molecular weights of the plasmids were determined using a DNA supercoiled ladder (Gibco BRL, USA).

Restriction endonuclease analysis of DNA. Total DNA from the selected strains of lactobacilli and bifidobacteria was isolated as described previously [29]. The chromosomal DNA was separated from the covalently closed circular forms by dye buoyant density centrifugation [28] in a CsCl gradient with ethidium bromide at 43000 rpm for 61 h. To achieve an additional purification from contaminating proteins, the preparations of bacterial DNA containing CsCl and ethidium bromide were incubated for 1 h at room temperature and then centrifuged at 8000 rpm for 20 min before the ultracentrifugation step. The chromosomal DNA obtained from ultracentrifugation was butanol extracted and dialysed against TE buffer. The DNA concentration was determined spectrophotometrically (O.D.₂₆₀). The DNA (0.75 µg) was digested for 6 h with 10 U of *EcoRI* (MBI Fermentas, Vilnius, Lithuania) in a 50 µl reaction mixture in buffer supplied by the manufacturer. After digestion, 7 µl of Ficoll loading buffer [29] and 5 µl of Tris-phosphate buffer were added to each sample, and DNA fragments were separated on 0.9% agarose (40V, 60 h) with cooling to about 15 °C. The gels were stained for 1 h with ethidium bromide (1.5 µg/ml) and destained in distilled water. The DNA fragments were visualized with a UV transilluminator and photographed. Molecular weights of the DNA fragments were calculated using the lambda DNA (MBI Fermentas) digested with *PstI* and lambda DNA digested with *Hind III*.

Because the only strain (*Lactobacillus plantarum* 18/4) that harbors plasmids was not isolated from any TD mice during *in vivo* experiments, total DNAs from the lactobacilli and bifidobacteria were used for the restriction analysis instead of the chromosomal DNAs. The conclusions on identity of the initial strains and the murine reisolates were made by comparison of positions of bands on the restriction endonuclease patterns of their total genomic DNAs.

In vitro susceptibility testing. All the strains of bifidobacteria and lactobacilli used were assayed for their resistance to 10 antibiotics. Minimal inhibitory concentrations (MIC) were determined in Wilson-Changren agar by the agar dilution method described previously [26].

Processing of specimens. Fecal samples from the mice were collected into disposable plastic Petri dishes; within 10 min, the specimens were weighed, homogenized, and diluted with prerduced Hanks' solution. To study the microflora of murine large bowel, 1-cm parts of the transverse colon were removed aseptically, the lumen contents were carefully pressed out onto sterile paper, and then processed as outlined above. The diluted samples were spread on the surface of the corresponding selective media for determination of the viable bacterial counts: Endo agar for enterobacteria; Enterococcus agar; Staphylococcus agar (Difco); Sabouraud Dextrose agar for yeasts; Schaedler agar (BBL, MD, USA) supplemented with 5% sheep blood, 0.01 g/l vit K, and 0.1 g/l kanamycin for anaerobic bacteria; MRS agar for lactic acid bacteria; and Bac-tofoc agar for bifidobacteria. Plates containing the latter three media were incubated anaerobically using GasPak Jars (BBL) at 37 °C for 2 days, while the other plates were incubated aerobically at 37 °C for 1-2 days. Mice were killed by cervical dislocation.

For the semiquantitative determination of bacteria in liver, about 500 mg of tissue were aseptically removed and homogenized with 2 ml prerduced saline. The undiluted specimens (0.1 ml) were spread onto Endo agar, Enterococcus agar, Staphylococcus agar, MRS agar, and Schaedler/vitK/kanamycin blood agar to detect the respective groups of microorganisms. The results were presented as the number of isolates of different types found in the liver and the incidences of translocation, determined by dividing specimens containing viable bacteria by the total number of samples tested.

Bacteria for animal administration. Bacteria for inoculation of the TD mice were obtained from overnight broth cultures, which were centrifuged for 15 min at 1000 g at 4 °C. The pellets were washed once with

sterile saline, and the bacterial concentrations were adjusted to the desired ones using turbidity standards.

Selection of Probiotic Bacteria *in Vitro* and *in Vivo*

Assays for inhibition of potential pathogens in vitro. Two methods were used to determine the ability of selected bacteria to inhibit potential pathogens:

- Agar deferred antagonism was performed exactly as described by Muriana and Klaenhammer [30]. Indicator layers were prepared by an addition of 100 μ l of overnight cultures of enterobacteria, enterococci, or staphylococci to 3.5 ml of BHI agar (0.7%). When testing the inhibition of the *Pseudomonas*, 1.0 ml of the overnight indicator's culture was added to 10.0 ml of sterile saline and the mixture poured onto the plates; after 5 min of incubation (room temperature), excessive liquid was evacuated. The diameters of clear and distinct inhibitory zones around the colonies of lactobacilli or bifidobacteria were measured after aerobic incubation for 14 hours at 37 °C.
- In the mixed culture method [31], lactobacilli or bifidobacteria and the indicator strains were incubated in 8 ml of the corresponding medium overnight at 37 °C. The concentration of microbial cells was then adjusted to ca. 3×10^8 per ml using McFarland standards. A 2-ml portion of broth taken from each of the tubes was mixed and incubated aerobically (in the case of lactobacilli testing) or anaerobically (bifidobacteria) at 37 °C. Samples from the mixed cultures were streaked at 0 and 24 h on MRS or Bactofoc agar and BHI agar. After anaerobic (MRS and Bactofoc plates) and aerobic (BHI) incubation, the microbial colony forming units (cfu) were counted. Inhibitory activity of the test microorganisms was presented as an Inhibitory Index calculated as a ratio between the initial and final concentrations of the indicator bacteria. Activity of the bifidobacteria against *Pseudomonas* was not determined by this method

because of the aerobic character of the indicator's growth.

Colonization abilities of lactobacilli and bifidobacteria strains. To determine the abilities of the lactobacilli and bifidobacteria strains to produce recognizable populations in the gastrointestinal tract, the test group (10 TD mice kept under GBI conditions) received by oral gavage 0.2 ml of the bacterial suspension containing ca. 1.0×10^9 cells once, one day after the decontamination was finished; 10 TD mice maintained in a separate isolator and given an equal volume of sterile saline served as a control for endogenous murine bacteria. The fecal viable counts of the bacteria were determined 5 days after the administration of the lactobacilli and bifidobacteria cultures. For each experiment, typical lactobacilli or bifidobacteria colonies were identified using microbiological methods and DNA analysis. All experiments were duplicated.

Antagonism of lactobacilli and bifidobacteria against potentially pathogenic bacteria in vivo. To estimate abilities of the lactobacilli and bifidobacteria strains (test organisms) to inhibit *Escherichia coli* K13, *Staphylococcus aureus* 209P, and *Streptococcus faecalis* 775 (indicator organisms) *in vivo*, 10 TD CBA mice maintained under GBI conditions were inoculated by oral gavage with 0.2 ml of a sterile saline suspension containing 1.0×10^9 cells of the test organism and 1.0×10^9 cells of one of the indicators, one day after the end of decontamination. Control group I consisted of 10 TD CBA mice maintained in isolators and given a suspension of the indicator organism only. To control for levels of the resident intestinal microflora, an additional control group II (10 TD mice given sterile saline) was used in each experiment. At day 3 after inoculation with bacteria, the mice were necropsied by cervical dislocation and the concentrations of lactobacilli or bifidobacteria as well as the indicator organisms were determined in the lumen contents of the large intestine. *In vivo* inhibitory properties of the test bacteria were estimated comparing the indicator's concentrations in the intestine of the test and control group I mice. All experiments were done twice.

Radiation

Mice were given whole-body gamma irradiation from bilaterally positioned ^{137}Cs sources in a dose of 7.0 Gy at a rate of 1.52 Gy/min. Mice were irradiated in aerated plexiglas containers. After irradiation, the mice were placed in either conventional conditions or gnotobiological isolators, where they were maintained during the next 30 days. Autoclaved cages, bedding, feed, and water were changed every other day. Experimental animals that received the different bacterial preparations (i.e., lactobacilli, bifidobacteria, lactobacilli/bifidobacteria, or saline) were housed in individual isolators.

Antimicrobial and Probiotic Therapy for Irradiated Mice

The effects of three antibiotics and their combination with probiotics on survival of irradiated mice were studied during the first set of experiments. All mice were placed into the gnotobiological isolators after irradiation. Lomefloxacin (Searle & Co., Chicago, IL), ciprofloxacin (Ferane, Moscow, Russia), and amikacin (Bristol-Myers Squibb Co, Syracuse, NY) were administered to the mice in 0.2 ml of sterile saline by an oral gavage daily in a dose of 50 mg/kg/day. Probiotics (*L5/4* or *B44*) were given in 0.2 ml of sterile saline by an oral gavage in a dose of ca. 10^9 cfu/mouse/day. The combined probiotic consisted of a mixture of 5.0×10^8 cfu of *L5/4* and 5.0×10^8 cfu of *B44* in 0.2 ml of sterile saline.

Two schemes of antibiotic administration were tested: Scheme 1, prior to probiotic administration, and Scheme 2, simultaneous with probiotics. In the first scheme, the antibiotics were administered from day 3 to day 5 postirradiation, followed by administration of the probiotics 8 hours and 24 hours after the last dose of the antibiotics was given. In the second scheme, the antibiotics were administered on days 1 through 7 postirradiation and the probiotics were given on days 1, 3, 5, 7, 9, 11, 13, and 15.

Each experiment consisted of 240 mice (16 groups of

15 animals). Each group received one of the probiotics (lactobacilli, bifidobacteria, or lactobacilli-bifidobacteria mixture, or saline as a control) and one of the following:

- ciprofloxacin
- lomefloxacin
- amikacin
- saline

Two different schemes of antibiotic/probiotic administration were examined. Three replicates of each experiment were done. A total of 1440 mice was used during 6 experiments. The mice were observed for mortality during 30 days postirradiation.

During the second set of experiments, the effect of the optimal antibiotic/probiotic treatment scheme (lomefloxacin combined with lactobacilli administered according to Scheme 2) on the intestinal and peripheral organ (liver) microflora was studied. Each experiment consisted of 240 mice. After irradiation, 120 mice (4 groups of 30 animals) were put into the isolators, where they received one of the following treatments:

- lomefloxacin plus lactobacilli
- lomefloxacin plus sterile saline
- sterile saline plus lactobacilli
- sterile saline alone

The other 120 mice formed analogous groups that were kept under conventional conditions.

Each group (30 mice) was subdivided into two groups of 15 mice: one group was observed for 30 days for survival, and the second group was used for microbiological studies. Intestinal microflora (large intestine) and the presence of microorganism translocation (liver cultures) were studied in five mice selected randomly from each such group on days 8 and 14 after irradiation.

Three replicates of the experiment were done. A total of 720 mice was used.

Statistical Analysis

Student's *t*-test was used in the statistical comparison of the results of antagonistic activity testing by the deferred antagonism method and in the comparison of the mean bacterial counts found in the experimental animals. All counts obtained from the mixed culture method were transformed into common logarithms. Counts less than detection level were excluded from calculation. The Wilcoxon T-test was used to estimate differences in the numbers of micro-

organisms before and after incubation in the mixed culture method; Wilcoxon U-test was used to compare differences in the Inhibitory Indexes for different test bacteria. Survival analysis was done using the Mantel-Cox test [32]. The Wilcoxon U-test was used in the statistical comparison of the mean survival times between experimental groups. The Fisher angle transformation test was employed to check the differences in qualitative characteristics (e.g., frequency of occurrence of a definite microorganism) between experimental groups.

Results

Identification and *in Vitro* Characterization of Probiotic Strains

Identification of freshly isolated microorganisms. All strains of lactobacilli used in this work meet the common criteria for genus *Lactobacillus*, that is, gram-positive nonsporing, microaerophilic, catalase-negative rods. These strains were identified at the species level according to their carbohydrate fermentation patterns. All strains of bifidobacteria were rods of various shapes with the typical morphology: gram-positive, nonspore-forming, nonmotile, anaerobic, indole negative, with F6PPK activity. These bifidobacteria were identified as *B. longum* (three strains), *B. adolescentis* (three strains), and *B. bifidum* (four strains) according to their carbohydrate fermentation profiles.

Plasmid contents of the lactobacilli and bifidobacteria strains. The strains most suitable for the next stages of work, that is, *L18/4*, *L5/4*, *BUX*, and *B44* (see table 1), were subjected to the plasmid isolation procedure. It was found that all the strains are free of plasmid DNA except *L18/4*, which harbors three plasmids (figure 1).

Restriction endonuclease analysis of the chromosomal DNA. Electrophoresis of *EcoRI*-digests of chromosomal DNA, extracted from strains *L18/4*, *L5/4*, *BUX*, and *B44* and allowed to obtain clearly recognized patterns, produced a suitable number of fragments: 28 for *L18/4*, 17 for *L5/4*, 19 for *B44*, and 18 for *BUX* (figure 2). The calculated lengths of the fragments are showed in table 2.

Antagonistic activity. The results of inhibitory activity examination using the deferred antagonism method are presented in tables 3 and 4. The lactobacilli strains showed marked differences in their ability to suppress

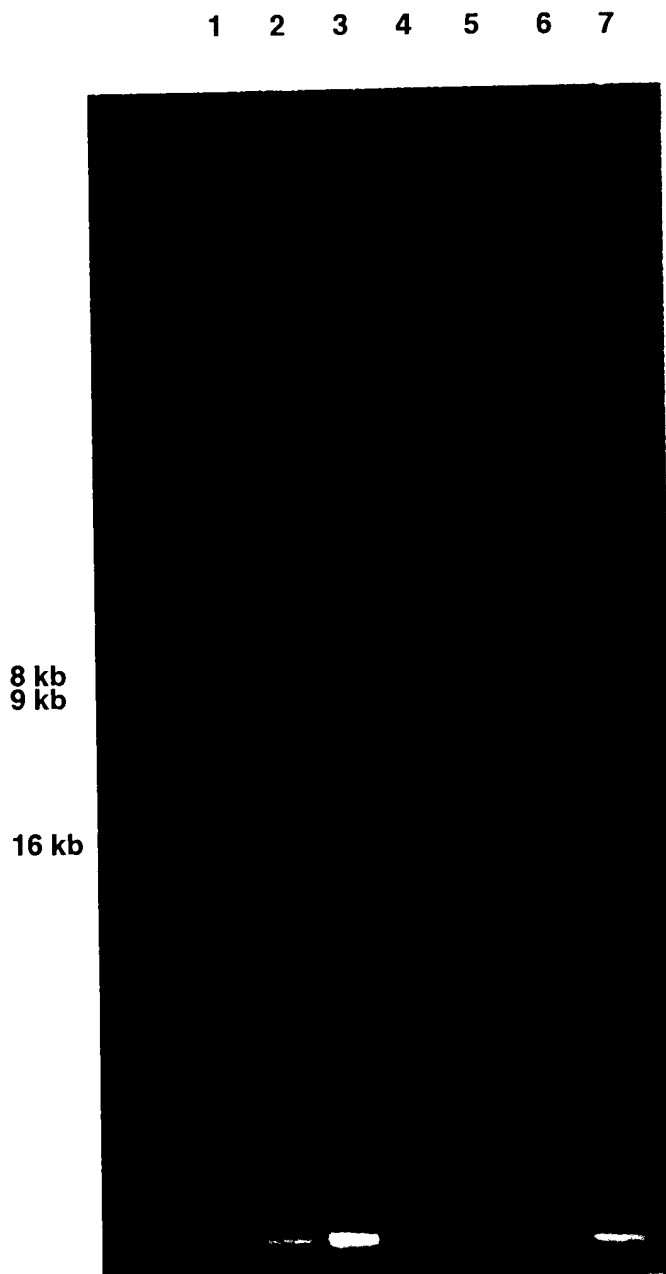


Figure 1. Plasmid content of the lactobacilli and bifidobacteria strains. Line 1, *L. acidophilus* 5/4; lines 2 and 7, DNA ladder (Gibco BRL); line 3, *L. plantarum* 18/4; line 4, *B. longum* 44; lines 5 and 6, derivatives of *L. acidophilus* 88 (used as the standards). The positions of covalently closed circular forms of *L. plantarum* 18/4 plasmids are indicated.



Table 2. Number of nucleotide base pairs in fragments observed in the *EcoRI*-digests of chromosomal DNA of lactobacilli and bifidobacteria

Bacterial strain			
<i>L18/4</i>	<i>L5/4</i>	<i>B44</i>	<i>BUX</i>
15233	11966	17892	10151
13501	10706	13501	9455
12968	9125	10898	8965
11966	8652	10151	8652
11494	8350	9972	8059
11292	7917	9455	7778
10898	7641	8965	7641
10706	7507	8652	7375
10518	7245	8350	7245
9625	6992	8059	6992
9455	6630	7778	6869
9125	6399	7675	6630
8652	5960	7118	6513
8204	5855	6630	6286
8059	5651	6286	6067
7917	5358	5960	5752
7507	5080	5752	5551
7245		5454	5264
6992		5264	
6748			
6513			
6286			
6067			
5752			
5651			
5454			
5358			
5171			

similar to those of *L5/4* against *E. coli* and *S. aureus* ($p > 0.05$) and of *L18/4* against *E.coli* ($p > 0.05$), but significantly weaker inhibition of *K. ozaenae* and *S. faecalis* than *L5/4*, and of *K. ozaenae*, *S. faecalis*, and *P. aeruginosa* than *L18/4*. *K. ozaenae* was found to be the organism most sensitive to the action of lactobacilli.

Among the *Bifidobacterium* strains tested, variability in the inhibitory properties was also found, but it was less evident than for the *Lactobacillus* strains. Only

Figure 2. Agarose gel electrophoresis of the chromosomal DNA extracted from lactobacilli and bifidobacteria and digested with *EcoRI*. Lines 1 through 8: *B. longum* 44, *B. longum* 211, *B. longum* 211 (repeated), *L. acidophilus* 5/4, *L. acidophilus* 13 α , *B. adolescentis* UX, *L. plantarum* 18/4, *PstI*-restricted DNA from lambda phage.

the growth of potentially pathogenic microorganisms. Strains *L5/4* and *L18/4* demonstrated the highest inhibition capacities among the 10 *Lactobacillus* isolates examined. Strain *L25* revealed antagonistic activity

Table 3. Inhibition of indicator bacteria by *Lactobacillus* test strains in the deferred antagonism test

Indicator culture	Test organisms									
	L6/14	L8/14	L14/14	L20/14	L13	L26	L25	L5/4	L18/4	L25/4
<i>E. coli</i> K13	4.8±1.6 ^a	6.4±1.7	4.7±1.6	4.8±1.4	9.7±2.6	17.1±3.1	22.3±2.5	29.8±3.0	27.6±5.3	6.2±2.2
<i>K. ozaenae</i> K4	20.8±2.3	29.5±6.0	19.8±1.7	20.5±2.1	32.0±5.8	29.5±6.0	35.5±4.1	52.0 ^b	46.7±6.1	43.7±3.2
<i>S. aureus</i> 209P	5.3±1.4	6.1±1.7	6.5±2.2	4.4±1.5	8.5±3.3	17.5±4.6	20.0±2.4	35.3±4.0	30.8±3.6	7.7±1.4
<i>S. faecalis</i> 775	3.3±1.1	4.0±1.0	3.3±0.5	3.1±0.6	5.7±0.8	9.6±1.7	10.1±1.5	21.7±1.2	22.3±3.2	2.8±2.4
<i>P. aeruginosa</i> 27853	9.0±3.5	6.7±4.2	7.1±2.0	11.6±4.7	15.3±4.5	6.8±1.1	17.5	18.0	26.0±11.9	8.0

^a Mean ± SD of diameters of inhibitory zones^b Mean of diameters of inhibitory zones detected from 2-3 colonies of the test organism**Table 4.** Inhibition of indicator bacteria by *Bifidobacterium* test strains in the deferred antagonism test

Indicator culture	Test organisms									
	BUX	BVL	BS2	B44	B211	B213	B221	B235	B1/6	B4M
<i>E. coli</i> K13	16.4±2.2 ^a	9.2±1.6	11.6±2.5	10.7±3.0	10.9±2.5	3.8±0.8	4.1±0.8	3.5±0.6	2.7±0.5	10.8±3.1
<i>K. ozaenae</i> K4	40.4±1.8	18.3±3.4	37.5±3.8	17.5±2.7	19.1±5.2	8.7±2.6	5.0±1.6	8.0±1.1	8.7 ^b	28.0±5.8
<i>S. aureus</i> 209P	15.7±3.1	9.2±1.6	9.0±1.9	10.0±2.1	8.9±2.2	2.8±0.8	3.7±1.1	1.9±0.9	2.0	10.5±2.0
<i>S. faecalis</i> 775	10.1±1.4	6.2±2.9	6.9±1.4	6.3±1.0	2.3±0.5	1.7±1.1	2.1±0.4	2.3±0.5	0 ^c	6.5±0.9
<i>P. aeruginosa</i> 27853	18.5±2.5	9.5±1.8	11.7±3.4	9.5±1.8	10.5±2.4	6.5±2.4	9.6±3.8	5.4±0.6	0	12.2±3.0

^a Mean ± SD of diameters of inhibitory zones^b Mean of diameters of inhibitory zones detected from 2-3 colonies of the test organism^c No inhibitory zones were detected.

strain BUX developed the wide inhibitory spectrum that included *S. faecalis*. Strains BVL, BS2, B44, B4M, and partially B211 formed a second group according to their activity: these organisms have lower activities than BUX against the indicator cultures (only strain BS2 inhibits *K. ozaenae* to the same extent [$p > 0.05$]), but, in general, they suppress the potential pathogens more actively than the other four strains.

The strong inhibitory properties of strains L5/4, L18/4, and BUX were confirmed by the mixed culture method (tables 5 and 6), where the differences among the strains examined became more evident. Plates streaked with the mixed culture at 0 h showed good growth (10^8 – 10^9 cfu/ml) of the indicator microorganisms as well as the strains of lactobacilli or bifidobacteria. After 24 h of incubation of the mixtures containing L5/4 or L18/4, a decrease in the colony

counts of *E. coli*, *K. ozaenae*, *S. aureus*, and *P. aeruginosa* to less than 10^2 cfu/ml was found. In the same time (24 h), the counts of indicator microorganisms in the control samples (without test bacteria) increased to 10^9 – 10^{10} cfu/ml. The greatest ability to inhibit *S. faecalis* in the mixed cultures was observed in strains L13 and L14/14. On the other hand, some strains showed no inhibitory influence on the growth of the indicator microbes. All strains of lactobacilli except L18/4 and L8/14 continued to grow in the test tubes.

Unlike the lactobacilli, no additional growth of the bifidobacteria was recorded after 24 h of incubation of the mixture cultures. When mixed with the indicators, both strains B44 and BS2 demonstrated the highest indexes of inhibition of *E. coli* and *S. aureus* compared to the other bifidobacteria ($p < 0.05$): concentration of the indicators lowered to 10^5 – 10^6 cfu/ml. Strains B44

Table 5. Effect of *Lactobacillus* test strains on growth of indicator bacteria in mixed cultures

Indicator culture	Test organisms									
	L6/14	L8/14	L14/14	L20/14	L13	L26	L25	L5/4	L18/4	L25/4
<i>E. coli</i> K13	0.96 (0.82-0.99) ^a	2.08 ^b	NG ^c	NG	NG	1.00 (0.97-1.02)	1.33 (1.30-1.35)	NG	NG	NG
<i>K. ozaenae</i> K4	1.00 (0.98-1.02)	4.52 (4.15-4.39)	4.34	NG	3.29	0.95 (0.93-0.99)	NG	NG	NG	NG
<i>S. aureus</i> 209P	0.94 (0.89-1.00)	2.53 (1.80-3.23)	2.09 (1.78-2.32)	NG	NG	0.91 (0.87-0.96)	1.57 (1.41-1.67)	NG	NG	1.75 (1.51-2.00)
<i>S. faecalis</i> 775	0.89 (0.81-0.98)	1.13 (1.08-1.19)	1.31 (1.20-1.37)	1.07 (1.01-1.15)	1.39 (1.29-1.42)	0.97 (0.89-1.14)	1.16 (1.09-1.24)	1.11 (1.0-1.16)	1.16 (1.12-1.19)	1.06 (0.98-1.19)
<i>P. aeruginosa</i> 27853	0.95 (0.89-1.02)	NG	NG	NG	NG	0.99 (0.84-1.08)	1.67 (1.47-1.96)	NG	NG	NG

^a Mean value (range) of Inhibitory Indexes^b Value obtained from 1-2 experiments, where growth of the indicator organism was detected**Table 6.** Effect of *Bifidobacterium* test strains on growth of indicator bacteria in mixed cultures

Indicator culture	Test organisms									
	BUX	BVL	BS2	B44	B211	B213	B221	B235	B1/6	B4M
<i>E. coli</i> K13	1.34 (1.27-1.36) ^a	1.30 (1.24-1.35)	1.71 (1.40-2.26)	1.49 (1.24-1.63)	1.19 (1.14-1.23)	1.21 (1.12-1.36)	1.03 (0.98-1.10)	1.10 (1.01-1.20)	1.16 (1.08-1.22)	1.27 (1.2-1.33)
<i>K. ozaenae</i> K4	2.99 ^b	NG ^c	NG	NG	4.30	NG	4.33	NG	NG	NG
<i>S. aureus</i> 209P	1.47 (1.44-1.57)	1.43 (1.23-1.60)	1.55 (1.38-1.70)	1.92 (1.56-2.19)	1.39 (1.28-1.49)	1.53 (1.43-1.59)	1.42 (1.22-1.55)	1.42 (1.32-1.57)	1.34 (1.21-1.53)	1.44 (1.39-1.47)
<i>S. faecalis</i> 775	1.10 (1.05-1.17)	0.95 (0.93-0.97)	1.07 (1.05-1.10)	1.04 (1.02-1.07)	0.97 (0.90-1.01)	1.01 (0.97-1.04)	1.07 (1.06-1.07)	1.00 (0.97-1.02)	1.01 (0.98-1.05)	1.01 (0.97-1.05)

^{a,b,c} See table 5 footnotes.

and BS2 were also active against *K. ozaenae* and *S. faecalis*. Meanwhile, no statistically significant differences were found comparing these strains to each other ($p > 0.05$). Strain BUX also possesses high inhibitory indexes by this test.

Thus, the strains of lactobacilli L5/4 and L18/4 as well as bifidobacteria BUX, B44, and BS2 have high activities in both interference tests, so that they can be considered as suitable for *in vivo* studies. During the further experimental stages, we used strains L5/4, L18/4, BUX, and B44.

Susceptibility of the *Lactobacillus* and *Bifidobacterium* isolates to antibiotics. The MICs of antibiotics for lactobacilli and bifidobacteria strains used in this work are shown in table 7. All the strains examined were found to be resistant to lomefloxacin; a majority of the strains showed resistance to ciprofloxacin and gentamicin. The lactobacilli and bifidobacteria were susceptible to penicillin G (except L18/4), ampicillin, and erythromycin. A majority of the strains were susceptible to oxacillin and cephalosporins. Sensitivity to tetracycline and streptomycin varied from strain to strain. Strain L18/4 was highly resistant to penicillin G and

Table 7. Antibiotic susceptibilities of the *Lactobacillus* and *Bifidobacterium* strains

Strain	Minimal inhibitory concentration ($\mu\text{g/ml}$)									
	P	Ox	Amp	Kz	Str	Tc	Em	Cn	Cfp	Lom
L6/14	<1	2	<1	<1	>200	4	<1	8	64	32
L8/14	<1	2	<1	<1	>200	8	<1	8	64	32
L14/14	<1	2	<1	<1	32	4	<1	8	64	16
L20/14	<1	2	<1	<1	>200	4	<1	8	64	16
L13	<1	2	<1	<1	32	128	<1	16	64	16
L26	<1	32	<1	4	32	16	<1	2	8	16
L25	<1	32	<1	4	32	16	<1	1	8	16
L5/4	<1	2	<1	<1	32	8	<1	16	32	64
L18/4	128	128	4	32	16	64	<1	2	64	64
L25/4	<1	<1	<1	<1	32	8	<1	32	32	64
BUX	<1	4	<1	16	8	128	<1	16	8	32
BVL	<1	<1	<1	ND	16	4	<1	8	8	8
BS2	<1	4	<1	16	16	4	<1	8	8	32
B44	<1	<1	<1	8	8	4	<1	8	8	64
B211	<1	2	<1	8	2	16	<1	8	16	64
B213	<1	<1	<1	4	8	16	<1	64	8	128
B221	<1	<1	<1	4	8	16	<1	64	8	128
B235	<1	<1	<1	4	8	16	<1	64	8	128
B1/6	<1	<1	<1	<1	8	128	<1	64	8	128
B4M	<1	<1	ND	2	64	128	ND	ND	ND	ND

P, penicillin G; Ox, oxacillin; Amp, ampicillin; Kz, cephalosporin; Str, streptomycin; Tc, tetracycline; Em, erythromycin; Cn, gentamicin; Cfp, ciprofloxacin; Lom, lomefloxacin; ND, not done

oxacillin, and also resistant to cephalosporin, tetracycline, and streptomycin, but not to erythromycin. The resistances of L5/4 to streptomycin, gentamicin, and ciprofloxacin were recorded. Bifidobacteria of strain BUX showed high resistance to tetracycline; strain B44 was resistant only to lomefloxacin.

table 8. Strain L5/4 possesses a capacity (relatively low) of producing detectable populations in the feces of some mice up to day 5 after a single administration: 4 of 19 mice tested during two analogous experiments carried lactobacilli completely identical to the original ones (figure 3). The second strain, L18/4, was not found in the feces of any animals.

Properties of the *Lactobacillus* and *Bifidobacterium* Strains *in Vivo*

Colonization. The results of the quantitative examination of feces of TD mice maintained under GBI conditions and given strains L5/4 and L18/4 are shown in

The presence of lactobacilli that differed strongly from the initial strains according to their cultural, morphological, and biochemical properties and plasmid contents were detected in both groups of animals (i.e., those given L5/4 or L18/4). Besides that, the appearance of *Lactococcus lactis* ssp. *lactis* in the feces of

Table 8. Bacterial counts in the feces of TD mice maintained under GBI conditions 5 days after single oral administration of *Lactobacillus* strains

Microorganisms recovered	Strain administered							
	<i>L. acidophilus</i> 5/4				<i>L. plantarum</i> 18/4			
	Test group ^a		Control group ^b		Test group		Control group	
Lactobacilli identical to the administered strain	4.9±2.0 ^c	4/19 ^d	<2.0	0/20	<3.0	0/20	<2.0	0/20
Other lactobacilli	6.5±0.9	5/19	<2.0	0/20	7.9±1.1	5/20	<2.0	0/20

^a TD mice colonized with either *L. acidophilus* 5/4 or *L. plantarum* 18/4^b TD mice given sterile saline instead of lactobacilli^c Mean ± SD of log₁₀ viable bacterial counts^d Number of mice yielding organisms/number of mice examined**Table 9.** Bacterial counts in the feces of TD mice maintained under GBI conditions 5 days after single oral administration of *Bifidobacterium* strains

Microorganisms recovered	Strain administered							
	<i>B. adolescentis</i> UX				<i>B. longum</i> 44			
	Test group ^a		Control group ^b		Test group		Control group	
Bifidobacteria identical or derived from the administered strain	6.6±1.1 ^c	16/20 ^d	<2.0	0/20	5.5±1.8 M44/17 ^e	14/19	<2.0	0/20
					7.6±0.4 M44/89 ^e	17/19	<2.0	0/20
Other bifidobacteria	<3.0	0/20	<2.0	0/20	<3.0	0/20	<2.0	0/20

^a TD mice colonized with either *B. adolescentis* UX or *B. longum* 44^b TD mice given sterile saline instead of bifidobacteria^c Mean ± SD of log₁₀ viable bacterial counts^d Number of mice yielding organisms/number of mice examined^e See text.

TD mice, contaminated by the lactobacilli, was registered frequently. By contrast, these organisms were never found in the control group animals.

As shown in table 9, in the groups receiving cultures of BUX or B44, high bifidobacterial counts were found in the feces of a majority of the mice at day 5 after administration. In the control mice receiving sterile saline instead of bacteria, no bifidobacteria were recorded.

Bifidobacteria were found in the feces of all animals receiving B44 5 days prior to the study. Two variants of bifidobacteria were observed: the first one, desig-

nated M44/89, was the only bifidobacteria type in 5 of the 19 mice examined. Two other mice harbored another bifidobacteria designated as M44/17. In the remaining 12 mice, both M44/89 and M44/17 were found, with a higher proportion of the former variant. These *Bifidobacterium* spp. isolates demonstrated the same carbohydrate fermentation patterns but different colony morphologies and antibiotic susceptibilities: M44/17 was found to be susceptible to tetracycline (tc^r) similar to the original strain (MICs = 4 µg/ml), while M44/89 was resistant to this antibiotic (MIC = 128 µg/ml, tc^r). Like B44, no plasmids were found in either isolate. Moreover, we failed to find any differences between the restriction endonuclease patterns of



Figure 3. Comparative electrophoretic analysis of *EcoRI*- digests of the total DNA extracted from the initial lactobacilli and bifidobacteria strains or from their reisolates from the intestine of TD mice. Line 1, *HindIII*-digested DNA of lambda phage; line 2, *B. adolescentis* UX; line 3, BUX/53 (reisolate of *B. adolescentis* UX); line 4, *B. longum* 44; line 5, M44/89 (*tc^r* reisolate of *B. adolescentis* UX); line 6, M44/17 (*tc^s* reisolate of *B. adolescentis* UX); line 7, *L. acidophilus* 5/4; line 8, L5/4/64 (reisolate of *L. acidophilus* 5/4).

the genomic DNAs extracted from these bacteria (figure 3). In all probability, the appearance of strain M44/89 can be explained as a mutation of B44, which led to acquisition of the resistance to tetracycline. Therefore, the results suggest that strain B44 together

with its derivatives existed in the feces of the monoassociated TD mice in high counts (10^7 – 10^8 cfu/g) up to day 5 postinoculation.

Strain BUX, administered to 20 TD mice, colonized the intestines of 16 of them at levels ranging from 10^4 to 10^7 cfu/g. Typical isolates demonstrated complete identity with the original strain in their susceptibility patterns and ability to utilize carbohydrates; plasmids were not registered in these isolates.

In vivo antagonistic activities of the lactobacilli and bifidobacteria. Both L5/4 and *L. plantarum* 18/4 demonstrated inhibitory activities against the strains of *E. coli* and *S. aureus* when administered to the TD mice simultaneously with the indicator. The antagonism resulted in a statistically significant decrease in the mean viable counts of the indicator microorganisms in the large intestine of the mice. The degree of *S. aureus* inhibition was virtually the same for both test strains of lactobacilli. For *E. coli*, this effect was more pronounced in the mice receiving strain L5/4: the resultant mean concentration of the indicator was approximately 3 logs lower than in the control group I animals (sterile saline instead of lactobacilli). Nevertheless, L5/4 had no effect on the frequency of *E. coli* found in the test animals, in contrast to L18/4 (table 10). Inhibition of *S. faecalis* was registered only by the action of L18/4, not L5/4.

In the mice receiving strains L18/4 or L5/4 together with the indicator bacteria, lactobacilli were isolated from the intestine of 12 mice of a total 59 mice

Table 10. *In vivo* interaction between test strains of lactobacilli and indicator microorganisms

Groups ^a	Indicator organism		Lactobacilli	
	Intestinal population level ^b	% pos ^c	Intestinal population level	% pos
<i>L. acidophilus</i> 5/4				
<i>E. coli</i> :				
Test group	5.4±1.5 ^d	90	6.8±0.6	45
Control group I	8.4±0.6	85	<4.0	0
Control group II	4.2 ^e Eb	10	<4.0	0
<i>S. aureus</i> :				
Test group	3.4±0.4 ^d	60	5.8±1.3	40
Control group I	4.6±0.8	100	4.9±1.0	30
Control group II	3.0 St	46	4.5±0.5	25
<i>S. faecalis</i> :				
Test group	8.6±0.3	95	7.0±1.6	58
Control group I	8.5±0.4	100	5.2±0.1	15
Control group II		0	4.9±0.2	15
<i>L. plantarum</i> 18/4				
<i>E. coli</i> :				
Test group	8.3±0.5 ^d	40	4.2±0.4	15
Control group I	9.2±0.1	50		0
Control group II	4.5 Eb	10		0
<i>S. aureus</i> :				
Test group	4.1±1.8 ^d	68	4.0	5
Control group I	5.8±0.9	65		0
Control group II		0		0
<i>S. faecalis</i> :				
Test group	7.9±0.6	100	4.8±0.5	40
Control group I	8.4±0.5	100	<4.0	0
Control group II	<2.0	0	<4.0	0

^a Test groups consisted of TD mice receiving a mixture of the test and indicator organisms; control group I consisted of animals receiving the indicator organism only; animals of control group II received sterile saline instead of bacteria.

^b Mean ± SD of log₁₀ viable bacterial counts

^c Number of mice yielding organisms/number of mice examined x 100

^d Statistically significant at the p<0.05 level when compared with the mean values obtained from the control group I animals

^e Detected in only 1 or 2 mice

Eb, total enterobacteria; St, total staphylococci

examined (concentration range: 10⁴–10⁵ cfu/g) and from 28 of 59 mice (10⁴–10⁸ cfu/g), respectively.

The results of antagonistic activity testing of the *Bifidobacterium* strains are shown in table 11. Statistically significant decrease in the intestinal population level of *E. coli* compared with control group I was

Table 11. *In vivo* interactions between test strains of bifidobacteria and indicator microorganisms

Groups ^a	Indicator organism		Bifidobacteria	
	Intestinal populations level ^b	% pos ^c	Intestinal population level	% pos
<i>B. adolescentis</i> UX				
<i>E. coli</i> :				
Test group	8.3±0.7	90	4.2±0.5	20
Control group I	8.2±1.0	79	<3.0	0
Control group II	4.8±0.6 Eb	21	<3.0	0
<i>S. aureus</i> :				
Test group	4.2±1.2 ^d	100	5.8±1.3	40
Control group I	6.6±0.6	70	<3.0	0
Control group II	<2.0	0	<3.0	0
<i>S. faecalis</i> :				
Test group	8.9±0.1 ^d	100	6.9±1.4	45
Control group I	9.1±0.1	100	<3.0	0
Control group II	4.1±0.4	78	<3.0	0
<i>B. longum</i> 44				
<i>E. coli</i> :				
Test group	7.2±1.3 ^d	81	6.4±0.8	44
Control group I	9.1±0.8	95	<3.0	0
Control group II	4.0±0.3 Eb	46	<3.0	0
<i>S. aureus</i> :				
Test group	3.9±0.6 ^d	75	5.9±1.1	30
Control group I	7.3±1.2	100	<3.0	0
Control group II	<2.0	0	<3.0	0
<i>S. faecalis</i> :				
Test group	8.2±0.1	95	5.2±0.5	32
Control group I	7.9±0.1	100	<3.0	0
Control group II	<2.0	0	<2.0	0

^a Test groups consisted of TD mice receiving a mixture of the test and indicator organisms; control group I consisted of the animals receiving the indicator organism only; animals of control group II received sterile saline instead of bacteria.

^b Mean ± SD of log₁₀ viable bacterial counts

^c Number of mice yielding organisms/number of mice examined x 100

^d Statistically significant at the p<0.05 level when compared with the mean values obtained from the control group I animals

Eb, total enterobacteria; St, total staphylococci

recorded only after its coadministration with *B44*, but this strain had no inhibitory influence on the *S. faecalis* level. Strain *BUX* was capable of suppressing the streptococci population in the murine intestine. Both strains *B44* and *BUX* inhibited the *S. aureus* indicator, decreasing the mean indicator's concentrations by 4 (*B44*) and 2 logs (*BUX*).

Effects of Antibiotics and Probiotics in Combination With Gnotobiological Isolation on Survival

Saline-treated groups. Both schemes for administering ciprofloxacin and lomefloxacin provided significant increases ($p < 0.01$) in ultimate survival and mean survival time of CBA mice maintained under GBI following irradiation compared with control (saline plus saline) groups (table 12, figures 4 and 5). Amikacin increased the survival and the mean survival time only when administered from day 1 to day 7 after the irradiation (Scheme 2). When administered using Scheme 2, lomefloxacin showed the highest improvement in survival compared with ciprofloxacin or amikacin ($p < 0.05$). No statistically significant differences in survival were noted between the groups that received lomefloxacin using Scheme 1 or 2.

Lactobacilli-treated groups. When administered according to Scheme 2, L5/4 led to a statistically significant increase in the mean survival time ($p < 0.05$), but not in survival ($p > 0.05$) of the mice. Combination of the lactobacilli with either lomefloxacin, ciprofloxacin, or amikacin by Scheme 2 resulted in an improvement in survival and mean lifespan. Lomefloxacin was found to be more active than ciprofloxacin ($p < 0.05$). Lomefloxacin, but not the other antibiotics, also decreased mortality ($p < 0.05$) when administered prior to lactobacilli (Scheme 1).

Bifidobacteria-treated groups. Similar to the lactobacilli, treatment of irradiated mice with B44 by Scheme 2 increased the mean survival time (but not ultimate survival). All antibiotic/bifidobacteria combinations examined decreased mortality of the mice when administered from day 1 to day 7 (Scheme 2), but only

Table 12. Survival and mean survival times of irradiated mice maintained under GBI conditions according to the treatment schemes and therapy applied^a

Probiotic	Antibiotic	Treatment Scheme 1		Treatment Scheme 2	
		Survival, %	Mean survival time, days	Survival, %	Mean survival time, days
Lactobacilli	Ciprofloxacin	40	19.6	33*	19.2†
	Lomefloxacin	44*	21.8†	57*	23.9†
	Amikacin	16	14.9	49*	22.5†
	Saline	38	18.4	38	17.8†
Bifidobacteria	Ciprofloxacin	49*	20.9†	43*	23.2†
	Lomefloxacin	40	19.0†	56*	23.2†
	Amikacin	29	16.8	36*	20.3†
	Saline	18	15.6	30	17.7†
Lactobacilli plus bifidobacteria	Ciprofloxacin	39	19.0	52*	22.3†
	Lomefloxacin	36	19.9†	49*	21.0†
	Amikacin	50*	21.2†	25	16.9†
	Saline	20	15.0	24	16.0
Saline	Ciprofloxacin	50*	22.8†	46*	20.7†
	Lomefloxacin	51*	22.0†	68*	24.7†
	Amikacin	31	17.4	50*	21.8†
	Saline	26	16.3	24	15.1

^a See text.

* Differences are statistically significant compared with the saline-treated group according to the Cox-Mantel test.

† Differences are statistically significant compared with the saline-treated group according to the Wilcoxon U-test.

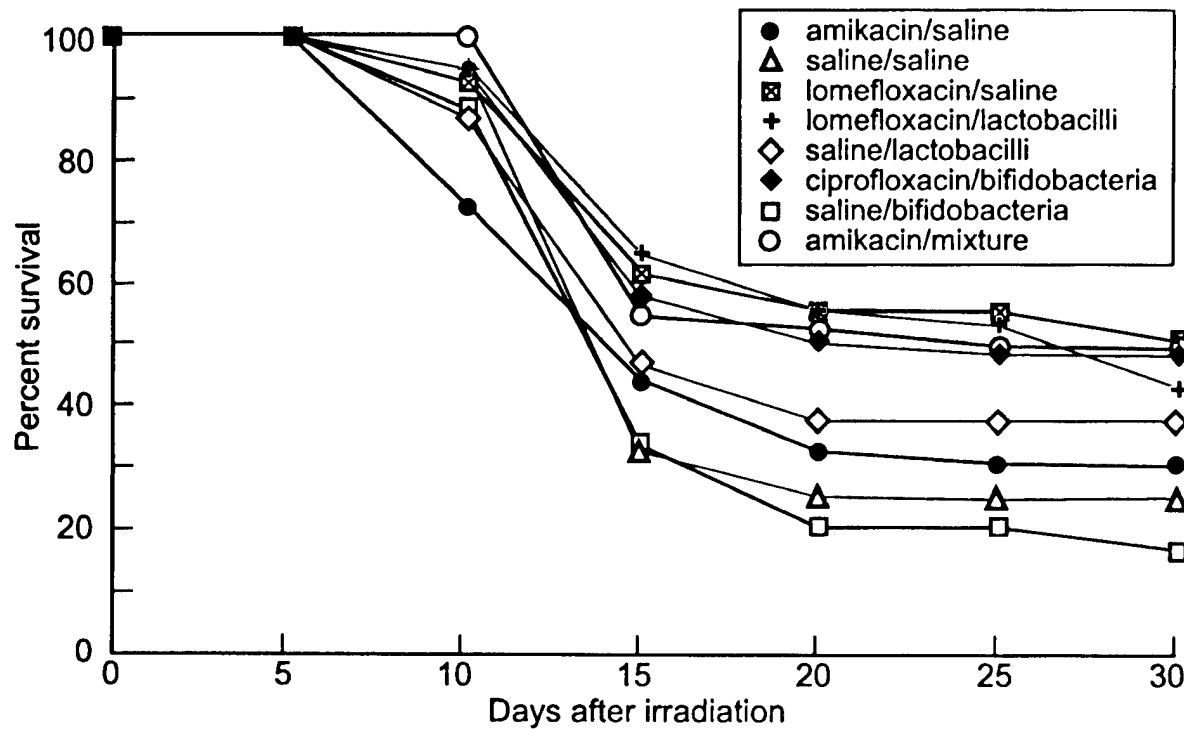


Figure 4. Survival of mice maintained under GBI conditions after irradiation with 7 Gy and receiving various treatments according to Scheme 1 (see text).

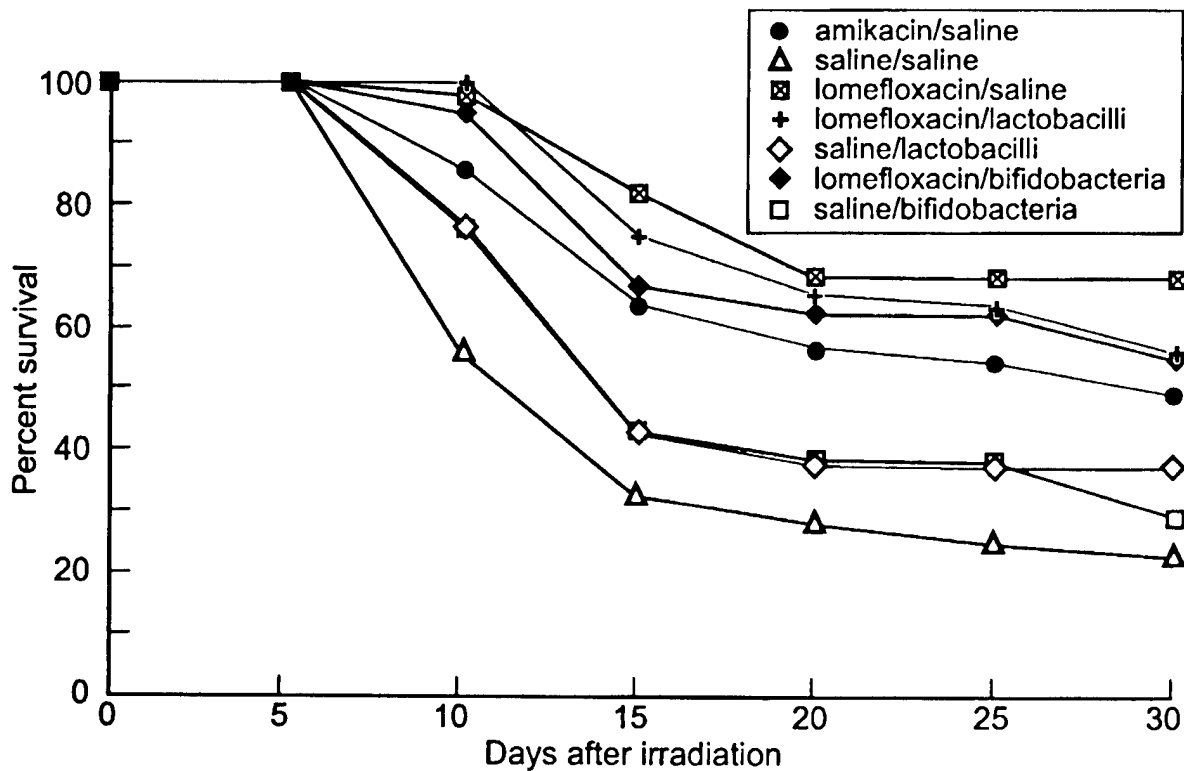


Figure 5. Survival of mice maintained under GBI conditions after irradiation with 7 Gy and receiving various treatments according to Scheme 2 (see text).

ciprofloxacin possessed such an effect after three-time administration (Scheme 1). There were no significant differences in survival between different antibiotic/bifidobacteria groups using Scheme 2 nor between the groups that received ciprofloxacin/bifidobacteria using Scheme 1 or Scheme 2.

Lactobacilli/bifidobacteria-treated groups. The mixed probiotic alone did not improve survival nor mean survival time of the irradiated mice, irrespective of the treatment regimen applied. Administration of the lactobacilli/bifidobacteria mixture after amikacin (Scheme 1) as well as concurrent treatment with ciprofloxacin or lomefloxacin (Scheme 2) demonstrated beneficial effects on both survival parameters considered. The lomefloxacin/bacterial mixture-treated groups (Scheme 2) had a prolonged mean survival time, but no better 30-day survival rates.

Effect of Lomefloxacin and *L. acidophilus* 5/4 on Survival Under Conventional and Gnotobiological Conditions

Administration of lomefloxacin in combination with lactobacilli, lomefloxacin alone, or lactobacilli alone significantly increased 30-day survival (91%, 82%, and 78%, respectively) as well as mean survival time (29.1 days, 27.7 days, and 26.7 days, respectively) of mice maintained in the isolators following irradiation compared with the saline-treated mice (44%, 20.3

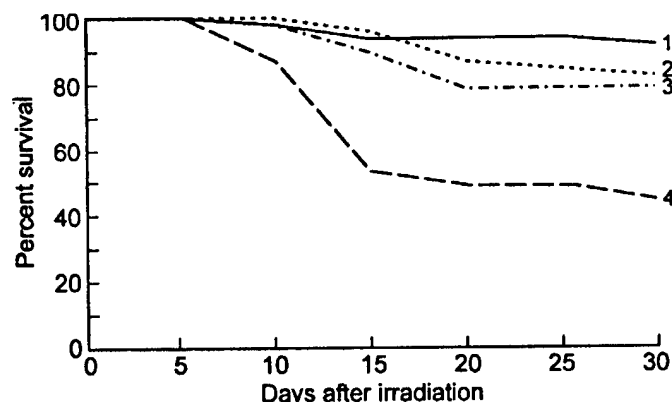


Figure 6. Survival of mice treated with lomefloxacin + lactobacilli (1), lomefloxacin (2), lactobacilli (3), or saline (4) under GBI conditions after irradiation with 7.0 Gy.

days) (figure 6). In mice maintained in conventional conditions after irradiation, the survival and the mean survival time increased in the lomefloxacin/lactobacilli-treated group (93%, 29.0 days), and in the lomefloxacin-treated group (84%, 27.8 days) compared with those in the saline-treated group (67%, 24.4 days), whereas administration of the lactobacilli alone increased only the mean survival time (73%, 25.4 days) (figure 7).

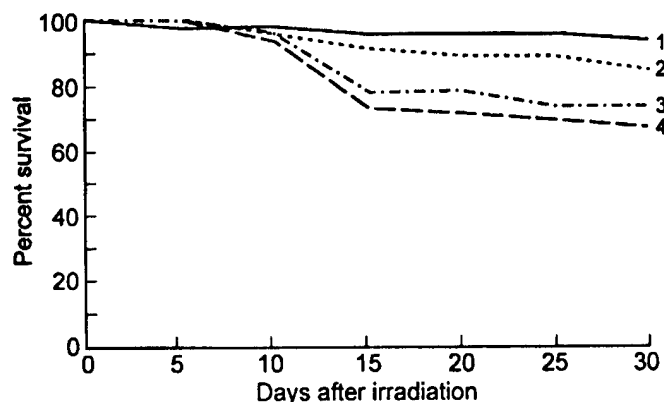


Figure 7. Survival of mice treated with lomefloxacin + lactobacilli (1), lomefloxacin (2), lactobacilli (3), or saline (4) under conventional conditions following irradiation with 7.0 Gy.

Intestinal Microflora in Irradiated Mice

GBI conditions after irradiation. Treatment of the irradiated mice with either lomefloxacin/lactobacilli combined or lomefloxacin alone led to a significant reduction both in the number and frequency of gram-negative aerobic and facultative anaerobic intestinal rods both at days 8 and 14 postirradiation when compared with normal mice (table 13). In the lactobacilli- and saline-treated mice, the counts of these bacteria were found to be the same as in the normal mice. Nevertheless, the former group demonstrated significantly lower levels of the bacteria compared to the latter one at both sampling times ($p < 0.05$). In the saline-treated group at day 14, frequency of isolation of lactose nonfermenting gram-negatives was significantly higher than in the normal mice (40.0% vs. 16.7%, $p < 0.05$).

In all experimental groups, the intestinal counts of enterococci and *Candida* yeasts as well were found to

Table 13. Intestinal microflora of the irradiated mice maintained under GBI and receiving different antibiotics and probiotics according to Scheme 2

Micro-organisms	8th day after irradiation				14th day after irradiation				Normal mice
	Lomefloxacin+ lactobacilli	Lomefloxacin	Lactobacilli	Saline	Lomefloxacin+ lactobacilli	Lomefloxacin	Lactobacilli	Saline	
<i>Bacteroides</i>	9.5±0.8 ^a 14/14 ^b	8.8±0.6 14/14	9.5±0.7 12/15	9.7±0.4 15/15	9.4±0.4 15/15	9.5±0.4 15/15	9.3±0.6 15/15	9.3±0.6 15/15	9.3±0.5 20/20
<i>Fusobacteria</i>	8.8±0.5 8/14	8.3±0.3 5/14	8.9±0.6 8/15	8.8±0.7 8/15	9.1±0.4 8/15	8.8±0.6 12/15	8.9±0.9 9/15	8.9±0.6 10/15	8.7±0.4 12/20
<i>Eubacteria</i>	8.9±0.5 8/14	8.4±0.6 7/14	8.9±0.9 11/15	9.3±0.6 12/15	8.8±0.5 14/15	9.1±0.8 10/15	9.2±0.7 9/15	8.7±0.6 11/15	8.9±0.6 12/20
<i>Peptococci</i>	8.3 ^c 1/14	<7.0	9.6 1/15	8.0 1/15	9.1±1.0 3/15	<7.0	<7.0	<7.0	8.7±1.2 4/20
<i>Peptostreptococci</i>	9.6 1/14	<7.0	<7.0	<7.0	<7.0	8.3 1/15	<7.0	<7.0	9.5 1/20
<i>Clostridia</i>	8.1 ^d 2/14	<7.0	<7.0	7.8±0.4 3/15	<7.0	8.3 1/15	7.8 1/15	<7.0	<7.0
Total strict anaerobes	9.7±0.7 14/14	9.0±0.4 14/14	9.8±0.6 12/15	9.9±0.4 15/15	9.7±0.3 15/15	9.7±0.5 14/15	9.6±0.6 15/15	9.6±0.3 15/15	9.5±0.5 20/20
<i>Lactobacilli</i>	8.7±0.9 15/15	8.8±0.4 15/15	9.1±0.6 15/15	8.8±0.6 15/15	8.7±0.8 15/15	8.6±0.6 15/15	9.2±0.7 15/15	8.7±0.6 15/15	9.2±0.7 20/20
Other LAB ^e	7.5±0.9 4/15	7.3 2/15	7.5±0.4 6/15	7.8±0.5 4/15	7.4±1.5 7/15	7.9±0.7 7/15	7.1±0.7 4/15	7.9±0.4 4/15	7.8±0.7 5/20
<i>Actinomyces</i> spp.	8.4±0.4 9/14	8.4±0.5 11/14	8.3±0.9 10/15	7.4±0.8 8/15	7.9±0.6 7/15	8.1±0.8 12/15	8.2±0.6 10/15	8.4±0.7 12/15	8.4±0.8 10/20
Total anaerobes	9.9±0.6 14/14	9.4±0.3 14/14	9.6±0.8 15/15	10.0±0.4 15/15	9.8±0.4 15/15	9.8±0.4 15/15	10.0±0.5 15/15	9.7±0.3 15/15	9.9±0.4 20/20
Gr (-) aerobic/facultative rods	6.6E 1/15	<6.0	7.5±0.9E 7/15 6.7Eb 1/15 7.9P 1/15 6.0Ac 1/15	8.2±1.0E 9/15 9.8Kl 1/15	<6.0	6.1S 2/15 6.7Ac 1/15	8.5±1.4E 7/15 6.3Pr 1/15 6.9Ac 1/15	8.6±1.4E 7/15 6.2Ac 2/15 7.5M 2/15 6.7Pr 1/15 6.3Kl 1/15	7.7±1.3E 18/30 7.3Eb 2/15 6.0Pr 2/15 8.0Cb 1/15
Total	6.6 1/15	<6.0	7.2±0.9 10/15	8.3±1.1 9/15		6.3 3/15	8.1±1.5 9/15	8.1±1.6 9/15	7.8±1.2 20/30
<i>Streptococci</i>	7.5±0.5 11/15	6.2±1.1 12/15	6.4±0.9 14/15	6.6±0.6 11/15	6.4±1.0 15/15	7.0±0.8 14/15	7.0±0.8 15/15	6.6±0.9 15/15	6.7±1.1 27/30
<i>Staphylococci</i>	2.7 1/15	2.7 2/15	2.9 1/15	4.2 1/15	<2.0	<2.0	2.3 1/15	<2.0	3.0±0.8 11/30
Total aerobes	6.8±0.7 13/15	6.4±1.0 12/15	7.1±0.9 14/15	7.9±1.1 14/15	6.4±1.0 15/15	6.9±0.1 15/15	7.8±1.2 15/15	7.6±1.4 15/15	7.7±1.1 27/30
<i>Candida</i> spp.	8.1±0.8 14/15	7.8±0.5 15/15	7.8±0.5 14/15	7.8±0.5 14/15	7.7±0.4 15/15	7.6±0.5 15/15	7.5±0.7 15/15	7.6±0.6 15/15	7.7±0.6 28/30

Ac, *Acinetobacter* spp.; Cb, *Citrobacter* spp.; E, *Escherichia coli*; Eb, *Enterobacter* spp.; Kl, *Klebsiella* spp.; M, *Morganella* spp.; P, *Pasteurella* spp.; Pr, *Proteus* spp.; S, *Serratia* spp.

^a Mean ± SD of log₁₀ number of viable microorganisms/g of intestinal contents

^b Frequency of occurrence (number of mice yielding the organisms/number of mice examined)

^c Mean log₁₀ number of viable microorganisms/g of intestinal contents

^d Log₁₀ number of viable microorganisms/g of intestinal contents

^e Total number of *Lactococcus* spp. and *Leuconostoc* spp.

be indistinguishable from those in the normal mice. In the irradiated mice kept under GBI conditions, staphylococci were isolated more rarely than in the normal mice ($p < 0.05$). Total aerobic counts were found to be significantly less in the mice receiving lomefloxacin/lactobacilli and lomefloxacin alone than in the normal and saline-treated mice on both days 8 and 14. In the lactobacilli-treated mice, these numbers were lower ($p < 0.05$) compared with the saline-treated (not the normal) mice on day 8 only.

In the animals receiving lomefloxacin (with or without the lactobacilli), the intestinal lactobacilli counts were the same as in the other groups. At the detection level used here (10^6 cfu/g), probiotic strain *L. acidophilus* 5/4 was not recovered from any mice.

In the lomefloxacin-treated mice, the total counts of strict anaerobes decreased at day 8 ($p < 0.05$), but increased again to those found in normal mice and in mice from the other groups by day 14. Increase in the strict anaerobes counts was noted in the saline-treated group at day 8, but not at day 14. These events connected primarily with changes in the counts of bacteroides. The intestinal counts of eubacteria, fusobacteria, and *Actinomyces* spp. in all experimental groups were found to be similar to those in the normal mice. Appearance of clostridia was registered in three mice from the saline-treated group at day 8 and eventually in the other groups.

Conventional conditions after irradiation. Similar to the mice maintained within gnotobiological isolators after irradiation, administration of lomefloxacin/lactobacilli as well as lomefloxacin alone markedly suppressed the intestinal gram-negative aerobic and facultative rods (table 14), whereas administration of lactobacilli alone did not cause such an effect. The mice treated with saline demonstrated a significant increase in the gram-negative counts compared with both normal and lactobacilli-treated mice at day 14 after irradiation. As a result, the total counts of intestinal aerobic bacteria in the saline-treated mice were significantly higher than in the normal mice or in the other experimental groups. No changes were recorded in the intestinal counts of enterococci, and *Candida*

yeasts in the animals of all experimental groups compared with the normal mice. Unlike the mice under GBI following irradiation, staphylococci were isolated with a frequency similar to those in normal mice.

In the mice receiving sterile saline after irradiation, the intestinal lactobacilli counts were significantly less than in the normal mice at both sampling dates. These counts remained the same as in the normal mice in the mice receiving lomefloxacin or lomefloxacin/lactobacilli. In the probiotic-treated group, the number of intestinal lactobacilli counts normalized by the end of treatment (day 14) after a transitory observed decrease at day 8. Lactobacilli identical to *L. acidophilus* 5/4 were not found in the mice at the detection level used.

The counts of strictly anaerobic bacteria were found to be increased in the saline-treated group at day 8, which was related to the appearance of clostridia and a slight increase of fusobacteria. Also, an increase in the total counts of strict anaerobes (mainly because of an increase in the bacteroides counts, $p < 0.05$) was found in mice treated either with lomefloxacin, lactobacilli, or saline compared with the normal mice at day 14 after irradiation. The intestinal counts of eubacteria and *Actinomyces* spp. had no changes compared with the normal control, but an increase in the numbers and frequency of fusobacteria ($p < 0.05$) was found in the lactobacilli-treated group in the second study. Clostridia were isolated from three mice of the saline-treated group at both days 8 and 14, and transitorily from animals of the other groups.

Translocation of Microorganisms in Irradiated Mice

A total of 103 microorganisms were isolated from livers of 240 irradiated mice (tables 15 and 16). The predominant organisms recovered were lactic acid bacteria (LAB), namely *Lactococcus* spp. and *Leuconostoc* spp. (26), and *Lactobacillus* spp. (13). Other frequent isolates (19) were also bacteria of the *Streptococcus* family: enterococci (12 isolates) and other streptococci (7). Enterobacteria were isolated from the livers in 11 cases: *Escherichia coli* (1 isolate),

Table 14. Intestinal microflora of the irradiated mice maintained under conventional conditions and receiving different antibiotics and probiotics according to Scheme 2

Micro-organisms	8th day after irradiation				14th day after irradiation			
	Lomefloxacin+ lactobacilli	Lomefloxacin	Lactobacilli	Saline	Lomefloxacin+ lactobacilli	Lomefloxacin	Lactobacilli	Saline
<i>Bacteroides</i>	9.5±0.5 ^a 13/14 ^b	9.4±0.7 14/14	9.6±0.7 15/15	9.0±0.5 15/15	9.5±0.5 15/15	9.9±0.4 14/15	9.8±0.5 15/15	9.8±0.7 14/14
<i>Fusobacteria</i>	9.2±0.6 6/14	8.8±0.4 6/14	8.8±0.6 11/15	9.9±0.4 10/15	8.9±0.7 6/15	8.8±0.8 10/15	9.2±0.5 14/15	9.0±0.6 12/14
<i>Eubacteria</i>	8.7±1.1 8/14	8.8±0.5 9/14	9.0±0.6 12/15	9.0±0.6 14/15	8.9±0.9 11/15	9.0±0.8 12/15	9.3±0.7 11/15	8.7±1.0 9/14
<i>Peptococci</i>	8.9 ^c 1/14	9.5 1/14	<7.0	8.4±0.2 3/15	<7.0	<7.0	<7.0	<7.0
<i>Peptostrep- toco</i>	7.4 1/14	7.5 1/14	9.8 ^d 2/15	8.6 1/15	<7.0	<7.0	9.4 1/15	8.0 2/14
<i>Clostridia</i>	7.6±0.8 3/14	8.0±0.0 4/14	7.0 1/15	8.0±0.6 3/15	7.7 1/15	<7.0	<7.0	7.5±0.5 3/14
Total strict anaerobes	9.6±0.6 14/14	9.6±0.5 14/14	9.8±0.6 15/15	9.8±0.4 15/15	9.7±0.5 14/15	10.0±0.4 15/15	10.0±0.5 15/15	9.9±0.6 14/14
<i>Lactobacilli</i>	9.1±0.4 15/15	8.9±0.8 15/15	8.8±0.5 15/15	8.7±0.5 15/15	9.4±0.8 15/15	9.2±0.5 15/15	9.3±0.7 15/15	8.6±0.4 14/15
Other LAB ^e	7.9 2/15	7.8±1.0 3/15	8.0±0.3 4/15	8.4±1.2 5/15	7.9 1/15	7.6±0.3 3/15	8.3±0.9 5/15	8.1±0.9 7/15
<i>Actinomyces</i> spp.	8.1±0.5 6/14	8.0±0.5 11/14	8.1±1.1 10/15	8.2±0.7 9/15	8.1±0.8 11/15	8.2±0.5 9/15	8.4±0.7 6/15	8.9±0.5 4/15
Total anaerobes	9.8±0.5 14/14	9.7±0.5 14/14	9.9±0.6 15/15	9.9±0.4 15/15	9.9±0.7 15/15	10.1±0.4 15/15	10.0±0.6 15/15	10.0±0.6 14/14
Gr (-) aerobic/facultative rods	<6.0	<6.0	7.5±0.9E 7/15 6.0Pr 1/15 6.0Eb 1/15	7.7±0.9E 10/15 8.8Ps 1/15 8.7Cb 3/15	<6.0	6.3Ac 1/15	7.7E 2/15 6.3Ac 2/15	9.6±1.1E 6/15 10.9Cf 1/15
Total	<6.0	<6.0	7.5±0.9 7/15	8.1±1.6 13/15	<6.0	6.3 1/15	9.9±1.1 4/15	9.9±1.2 6/15
<i>Streptococci</i>	6.7±0.6 12/15	6.1±0.8 13/15	6.3±1.4 11/15	6.8±0.7 13/15	6.2±1.0 15/15	6.5±0.8 13/15	6.4±1.1 13/15	6.8±1.3 11/15
<i>Staphylococci</i>	4.3 1/15	2.0 1/15	4.2±1.0 4/15	4.2±1.2 5/15	3.3±0.7 4/15	3.8±1.1 3/15	3.8±1.3 3/15	4.3±1.3 4/15
Total aerobes	6.7±0.5 12/15	6.4±0.6 12/15	7.2±0.8 12/15	7.8±1.0 14/15	6.2±0.9 15/15	6.6±0.8 13/15	6.3±1.5 15/15	8.1±2.0 12/15
<i>Candida</i> spp.	7.7±0.5 14/15	7.6±0.5 14/15	7.4±0.6 14/15	7.5±0.4 15/15	7.8±0.5 15/15	8.0±0.4 14/15	7.7±0.5 15/15	7.3±0.7 14/15

Ac, *Acinetobacter* spp.; Cb, *Citrobacter* spp.; E, *Escherichia coli*; Eb, *Enterobacter* spp.; P, *Pasteurella* spp.; Pr, *Proteus* spp.; Ps, *Pseudomonas* spp.

^a Mean ± SD of log₁₀ number of viable microorganisms/g of intestinal contents

^b Frequency of occurrence (number of mice yielding the organisms/number of mice examined)

^c Log₁₀ number of viable microorganisms/g of intestinal contents

^d Mean log₁₀ number of viable microorganisms/g of intestinal contents

^e Total number of *Lactococcus* spp. and *Leuconostoc* spp.

Table 15. Microorganisms isolated from livers of irradiated mice maintained under GBI conditions (specimens containing viable bacteria/total number of samples)

number of samples																	
Treatment group	Number of isolates														Incidence of translocation		
	Anaerobes				Aerobes												
	B	Eu	Pc	A	L	LAB	Ec	Eb	Cb	Ac	Sr	En	St	Mi		Cd	Total isolates
First study^a																	
Lomefloxacin+lactobacilli	0/15	0/15	1/15	1/15	0/15	2/15	0/15	0/15	0/15	0/15	0/15	2/15	1/15	0/15	0/15	7	3/15
Lomefloxacin	0/15	0/15	0/15	1/15	0/15	2/15	0/15	0/15	0/15	0/15	0/15	2/15	0/15	0/15	0/15	6	4/15
Lactobacilli	0/15	0/15	0/15	0/15	0/15	2/15	1/15	1/15	1/15	0/15	0/15	0/15	2/15	0/15	0/15	7	5/15
Saline	0/15	1/15	0/15	0/15	1/15	0/15	0/15	1/15	0/15	0/15	1/15	0/15	2/15	0/15	0/15	6	4/15
Total:	0/60	1/60	1/60	2/60	1/60	6/60	1/60	2/60	1/60	0/60	1/60	4/60	5/60	0/60	1/60	26	14/60
Second study																	
Lomefloxacin+lactobacilli	0/15	0/15	0/15	0/15	2/15	3/15	0/15	1/15	0/15	0/15	0/15	2/15	2/15	0/15	0/15	10	5/15
Lomefloxacin	1/15	1/15	0/15	1/15	2/15	3/15	0/15	0/15	0/15	0/15	1/15	2/15	1/15	0/15	0/15	12	5/15
Lactobacilli	0/15	0/15	0/15	0/15	0/15	2/15	0/15	0/15	0/15	0/15	1/15	2/15	0/15	1/15	0/15	6	3/15
Saline	0/15	0/15	0/15	1/15	0/15	1/15	0/15	0/15	0/15	1/15	1/15	0/15	1/15	0/15	0/15	5	4/15
Total:	1/60	1/60	0/60	2/60	4/60	9/60	0/60	1/60	0/60	1/60	3/60	6/60	4/60	1/60	0/60	33	18/60

B, bacteroides; Eu, eubacteria; Pc, peptococci; A, *Actinomyces* spp.; L, lactobacilli; LAB, lactic acid bacteria (other than lactobacilli); Ec, *Escherichia coli*; Eb, *Enterobacter* spp.; Cb, *Citrobacter* spp.; Ac, *Acinetobacter* spp.; Sr, *Streptococcus* spp.; En, enterococci; St, staphylococci; Mi, micrococci; Cd, *Candida* spp.

^a First study was done at day 8 postirradiation; second study was done at day 14 postirradiation.

Table 16. Microorganisms isolated from livers of irradiated mice maintained under conventional conditions (specimens containing viable bacteria/total number of samples)

Treatment group	Number of isolates															Incidence of isolates translocation	
	Anaerobes							Aerobes									
	B	Eu	Pc	Ps	A	L	LAB	Eb	Cb	M	Ac	Sr	En	St	Mi	Cd	Total
First study ^a																	
Lomefloxacin+lactobacilli	0/15	0/15	0/15	0/15	0/15	1/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	1
Lomefloxacin	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0
Lactobacilli	0/15	0/15	0/15	0/15	1/15	0/15	2/15	1/15	0/15	0/15	0/15	0/15	0/15	1/15	0/15	1/15	6
Saline	0/15	2/15	1/15	1/15	1/15	2/15	3/15	0/15	1/15	0/15	0/15	0/15	0/15	1/15	0/15	0/15	12
Total:	0/60	2/60	1/60	1/60	2/60	3/60	5/60	1/60	1/60	0/60	0/60	0/60	0/60	2/60	0/60	1/60	19
Second study																	
Lomefloxacin+lactobacilli	0/15	0/15	0/15	0/15	0/15	1/15	1/15	0/15	0/15	0/15	0/15	1/15	1/15	0/15	0/15	0/15	4
Lomefloxacin	1/15	0/15	0/15	0/15	0/15	1/15	1/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	0/15	3
Lactobacilli	1/15	0/15	0/15	0/15	0/15	3/15	2/15	0/15	0/15	1/15	0/15	1/15	0/15	1/15	0/15	0/15	9
Saline	2/15	0/15	0/15	0/15	0/15	0/15	2/15	0/15	1/15	0/15	1/15	1/15	1/15	0/15	1/15	0/15	9
Total:	4/60	0/60	0/60	0/60	0/60	5/60	6/60	0/60	1/60	1/60	1/60	3/60	2/60	1/60	1/60	0/60	25

B, bacteroides; Eu, eubacteria; Pc, peptococci; Ps, peptostreptococci; A, *Actinomyces* spp.; L, lactobacilli; LAB, lactic acid bacteria (other than lactobacilli); Eb, *Enterobacter* spp; Cb, *Citrobacter* spp.; M, *Morganella* spp.; Ac, *Acinetobacter* spp.; Sr, *Sireptococcus* spp.; En, enterococci; St, staphylococci; Mi, micrococci; Cd, *Candida* spp.

^a First study was done at day 8 postirradiation; second study was done at day 14 postirradiation.

Enterobacter spp. (4), *Citrobacter* spp. (3), *Morganella morganii* (1). *Acinetobacter* spp. was found in two mice (2 isolates). The other aerobic microorganisms recovered were *Staphylococcus* spp. (12 isolates) and *Micrococcus* spp. (2 isolates).

Anaerobic microorganisms recovered were bacteroides (5 isolates), *Eubacterium* spp. (4), *Peptococcus* spp. (2), *Peptostreptococcus* spp. (1), and *Actinomyces* spp. (6). Total numbers of anaerobic (including LAB and *Actinomyces* spp.) and aerobic isolates were 57 and 46, respectively. *Candida* yeasts were recovered on two occasions.

Incidences of translocation were 12/60 (20%) in the mice treated with lomefloxacin/lactobacilli and 11/60 (18.3%) in the mice treated with lomefloxacin, which were significantly less ($p < 0.05$) than in the saline-treated mice (20/60, 33%). In addition, the total numbers of the liver isolates in the lomefloxacin-treated mice were lower compared with the saline-treated mice. Although not statistically different from the saline-treated mice, the total number of isolates as well as the incidence of translocation were less in the lactobacilli-treated groups.

Translocation of the gram-negative enterics (*Enterobacter* spp.) to the liver of irradiated mice was registered in only one mouse receiving lomefloxacin alone or with lactobacilli, whereas lactobacilli had no influence (5 isolates) on it compared with the saline-

treated group (5 isolates). Translocation of strict anaerobes was observed more frequently in the saline-treated group (7 isolates) than in the other ones ($p < 0.05$).

No significant differences were found in the total number of aerobic isolates recovered from the saline-treated mice (14 isolates) and from the other groups, but this number was lowest in the lomefloxacin-treated mice (6 isolates).

The treatment regimens used had no influence on the numbers of *Streptococcus* spp. (calculated with or without *Lactococcus* spp. and *Leuconostoc* spp.), *Lactobacillus* spp., total LAB, and *Staphylococcus* spp. isolates. No differences were found in the incidences of translocation and the total number of liver isolates between the groups maintained under GBI and those under conventional conditions.

In the saline-treated mice, the number of liver isolates as well as the incidence of translocation reached their maximum by day 8, whereas in the other groups, the maximum was delayed up to day 14.

The probiotic strain *L. acidophilus* 5/4 did not translocate after being introduced into the mice. All lactobacilli strains recovered from the livers differed from the probiotic strain according to their biochemical properties or plasmid contents and restriction endonuclease patterns.

Discussion

Previously, it has been found that some strains of bifidobacteria and lactobacilli are capable of improving survival parameters in sublethally irradiated conventional mice when administered orally four times [33]. All three strains of bifidobacteria that provided beneficial effects on survival also demonstrated high degrees of antagonistic activity against enterobacteria by *in vitro* tests, while the correlation was not found in the case of lactobacilli. More recently, Bossart et al. [34] showed that *L. acidophilus* 11/83, which is antagonistically active against *Escherichia coli*, *Klebsiella pneumoniae*, and *Proteus mirabilis*, both *in vitro* and *in vivo* [23], also increases survival of conventional mice exposed to 7 Gy. In mice subjected to total antibiotic decontamination prior to irradiation with the same dose and then maintained in isolators, this lactobacilli strain also improved survival when administered seven times [22].

In the present study, we used strains of lactobacilli and bifidobacteria (*L. acidophilus* 5/4 and *B. longum* 44) that had been selected on the basis of their antagonistic properties *in vitro* and colonization abilities/inhibitory activities *in vivo*. In the first set of experiments, treatment of irradiated mice maintained under GBI conditions with these bacteria significantly increased mean survival time of the animals (but not the 30-day survival rate). The effect was registered only after a prolonged (eight times) course of the probiotics.

When combined with lomefloxacin, both bacteria as well as their mixture also demonstrated beneficial effects (indistinguishable from each other) on the outcome of acute radiation disease. Although these strains are both of human origin, in our minds, lactobacilli are more suitable for probiotic treatment in mice, as these organisms are generally recognized as indigenous for this animal species [14].

Repeat testing during the second set of experiments revealed that *L5/4* significantly increases both the survival rate and the mean survival time of irradiated mice maintained in isolators, but only the mean survival time when given to mice kept in conventional conditions following irradiation. Combined data from two sets of experiments show that the oral administration of *L5/4* increases survival of irradiated mice under GBI up to 58% compared with 36% for the saline-treated control ($p < 0.05$). Intestinal microflora analyses demonstrated that the lactobacilli-treated mice under GBI after irradiation had significantly lower counts of the gram-negative aerobic and facultatively anaerobic rods as well as the total aerobic counts than in the saline-treated mice during the entire course of the study. When the mice were kept under conventional conditions, this effect was registered only at day 14 after irradiation. Thus, gnotobiological isolation seems to facilitate the effect of the lactobacilli.

Previously, it has been found that *L. acidophilus* 11/83 administered to mice kept under GBI after irradiation leads to a strongly marked suppression in intestinal populations of enterobacteria, enterococci, and staphylococci accompanied by an increase in the number of lactobacilli [34]. In the present study, administration of *L5/4* to irradiated mice maintained the intestinal lactobacilli counts at normal levels at both sampling times (GBI conditions) and at day 14 (conventional conditions). Nevertheless, the degree of survival-promoting effects of these two strains (*L. acidophilus* 11/83 and *L5/4*) are comparable.

In our minds, these data demonstrate that the beneficial potential of probiotics is not only a function of their inhibitory properties. In theory, other factors, such as degree of immunostimulating activity, which varies greatly from one bacterial strain to another for lactobacilli [18] and bifidobacteria [35], or production of

bacteriocin-like substances [36] could also be involved. The strain used here is able to colonize the intestines of TD mice under GBI conditions at low levels and frequency. Irradiated conventional animals have much more complex intestinal microflora (including large populations of indigenous lactobacilli) than the TD mice, which creates the so-called phenomenon of colonization resistance so that successful colonization of the intestine with exogenous lactobacilli can hardly be expected.

It has been shown that irradiation of mice with 7.0 Gy from a ^{60}Co -source causes little or no bacterial translocation into the livers [1,8,12], while x irradiation at a dose of 6.75 Gy leads to bacteremias with streptococci [5]. In this study, we observed a massive translocation in 48 of a total 240 mice irradiated with 7.0 Gy. This difference can be explained by the use of another radiation source (^{137}Cs) and another strain of mice. The diet given can also affect translocation [37]. Here, lactic acid streptococci, namely *Lactococcus* spp. and *Leuconostoc* spp., together with enterococci and other streptococci were the microorganisms most frequently isolated from the liver. The other common isolates were *Lactobacillus* spp. (mostly *L. fermentum*), staphylococci, and strict anaerobes. Enterobacteria were recovered much more rarely than streptococci, which could be expected for the radiation dose used [5].

The present data confirm that the quinolones greatly reduce the translocation of enterobacteria and other gram-negative aerobic and facultative rods but fail to prevent the systemic spread of streptococci and other gram-positive bacteria in irradiated mice [1,7].

Bacterial translocation in gnotobiotic mice can be reduced by bacterial antagonism [38]. In the present work, it was found that the mice receiving L5/4 in either GBI or conventional conditions demonstrated a decrease in the translocation of strict anaerobes compared with the saline-treated mice. In addition, the probiotic tended to reduce the total number of liver isolates, the number of anaerobic isolates, and incidences of translocation.

Previously, it has been demonstrated that combinations of bifidobacteria with penicillins or aminoglycosides provide better treatment effects in irradiated mice than antibiotics alone [15,16]. Here, this synergism was observed only in mice receiving amikacin/bifidobacteria plus lactobacilli according to Scheme 1. The suggestion can be made that the effects of the quinolones, when these antibiotics were used by Scheme 2, exceeded those of the probiotics at days 8 and 14 postirradiation. Adding L5/4 to the treatment with lomefloxacin did not change the effects of the quinolone on the intestinal microflora and translocation of microorganisms. Meanwhile, the short course of the probiotics (Scheme 1) was not sufficient to improve survival.

Amikacin shows a lower activity than ciprofloxacin against intestinal enterobacteria and enterococci and additionally suppresses intestinal lactobacilli after oral administration to conventional mice under GBI conditions [39]. It is possible that administration of antagonistically active probiotic bacteria to irradiated mice after a course of amikacin could improve the decontamination effect of the antibiotic, preventing overgrowth of potential pathogens.

Therefore, the present study confirms the ability of lactobacilli and bifidobacteria to improve survival of sublethally irradiated mice, especially when they are kept under GBI conditions (for lactobacilli). Nevertheless, the strain of lactobacilli administered alone or in combination with lomefloxacin did not suppress streptococci and other intestinal gram-positive bacteria and did not prevent their translocation at day 8 and 14 postirradiation. In our minds, special attention should be paid to using bacteriocin-producing strains of lactobacilli in treatment of the infectious complications of acute radiation disease because some of these bacteriocins have a broad inhibitory spectrum *in vitro*, including against streptococci [36]. That suppression ability should be confirmed by *in vivo* experiments. Another important property to be considered in future studies is the immunomodulating activity of the probiotic bacteria.

Conclusions

- Live cultures of *L.acidophilus* 5/4 and *B.longum* 44 administered orally eight times increase survival of mice irradiated with 7.0 Gy and then maintained under gnotobiological isolation. The administration of *L. acidophilus* 5/4 helps to restrict overgrowth of the intestinal gram-negative aerobic and facultative rods, support the normal population levels of lactobacilli, and decrease translocation of the strictly anaerobic bacteria into the liver of irradiated mice. The translocation of *L. acidophilus* 5/4 was not registered.
- Gnotobiological isolation itself does not increase survival of conventional irradiated mice and has no influence on microorganism translocation but seems to assist the survival-promoting effect of the lactobacilli.
- The most frequent liver isolates recovered from the mice were lactic acid streptococci (*Lactococcus* spp. and *Leuconostoc* spp.). The other commonly recovered organisms were enterococci, lactobacilli, and staphylococci. Enterobacteria, *Acinetobacter* spp., strict anaerobes, *Actinomyces* spp., micrococci, and *Candida* spp. were isolated at relatively low frequencies.
- Being administered three or seven times, lomefloxacin and ciprofloxacin increase the survival rate and the mean survival time of mice kept under GBI following irradiation, while amikacin possesses such an effect only after seven administrations.
- Among the groups of mice receiving probiotics together with antibiotics, the highest survival rates were registered for combinations of *L. acidophilus* 5/4 or *B. longum* 44 with lomefloxacin.
- Lomefloxacin alone or when combined with *L. acidophilus* 5/4 greatly reduces both intestinal counts and frequency of translocation of gram-negative aerobic and facultatively anaerobic rods but not streptococci and other gram-positive bacteria into the liver of mice, irrespective of gnotobiological isolation.
- The optimum survival-promoting effects of antibiotics and probiotics occurs if the mixture of *L. acidophilus* 5/4 and *B. longum* 44 is administered after a short course of amikacin.
- Additional research efforts should be made to search for lactobacilli strains that selectively suppress streptococci and other gram-positive organisms, with particular attention to the ability to produce bacteriocin-like substances and to stimulate host immunity.

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